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Use of Chimeric Nectin-1(HveC)-Related Receptors to Demonstrate That Ability to Bind Alphaherpesvirus gD Is Not Necessarily Sufficient for Viral Entry

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Human nectin-1 (HveC, Prr1), a member of the immunoglobulin superfamily and a receptor for the entry of herpes simplex viruses 1 and 2 (HSV-1, HSV-2), pseudorabies virus (PRV), and bovine herpesvirus 1 (BHV-1), binds to viral gD. For HSV-1, HSV-2, and PRV, the gD-binding region of nectin-1 has been localized to the N-terminal V-like domain. To determine whether the two C-like domains of nectin-1 influenced gD binding and entry activity, genes encoding chimeric proteins were constructed. Portions of nectin-1 were replaced with homologous regions from nectin-2 (HveB, Prr2), a related protein with ability to mediate the entry of PRV, HSV-2, and Rid mutants of HSV-1, but not HSV-1 or BHV-1. Also, one or more domains of nectin-1 were fused to the two membrane-proximal Ig domains of CD4, a protein with no herpesvirus entry or gD-binding activity. The chimeric proteins were expressed in Chinese hamster ovary cells, which normally lack alphaherpesvirus entry receptors, and detected on the cell surface by one or more anti-nectin-1 monoclonal antibodies. One chimeric protein (nectin-1 amino acids 1–124 fused to CD4) failed to bind to soluble forms of HSV-1, HSV-2, PRV, and BHV-1 gD and, as expected, also failed to mediate entry of the viruses from which these gDs were derived. The other chimeric receptors bound all forms of gD. Some mediated the entry of all the viruses tested but others mediated entry of some but not all the viruses. We conclude that binding of gD to the nectin-1 V domain is not sufficient for entry activity, that there are structural requirements for entry activity independent of gD binding, and that these requirements are different for the several alphaherpesviruses that can use nectin-1 as a receptor.

Key Words: nectin-1; HveC; nectin-2; HveB; herpes simplex virus; pseudorabies virus; bovine herpesvirus 1; CD4; gD; entry.

INTRODUCTION

Certain alphaherpesviruses, such as herpes simplex viruses 1 and 2 (HSV-1 and HSV-2), porcine pseudorabies virus (PRV), and bovine herpesvirus 1 (BHV-1), can bind to cells through an initial interaction of viral gC or gB with heparan sulfate chains on cell surface proteoglycans. Following this binding, entry requires an interaction of viral gD with any one of several cell surface coreceptors. This interaction somehow triggers fusion between the viral envelope and cell membrane, an event which requires the activities of viral gB, gH, and gL, as well as gD and its receptor (reviewed by Spear, 1993; Spear *et al.,* 2000).

Human nectin-1 (Takahashi *et al.,* 1999), also known as herpesvirus entry protein C (HveC) (Geraghty *et al.,* 1998) or poliovirus receptor-related protein 1 (Prr1) (Lopez *et al.,* 1995), is one of the several gD-binding entry receptors for HSV-1 and HSV-2 and can also mediate entry of PRV and BHV-1 as well as bind to PRV and BHV-1 forms of gD (Cocchi *et al.,* 1998a, 1998b; Connolly *et al.,* 2001; Geraghty *et al.,* 2000, 1998; Krummenacher *et al.,* 1998). Nectin-1 is a member of the immunoglobulin (Ig) superfamily and is highly conserved in mammals, at least in the Ig-like domains of the ectodomain. Pairwise comparisons of the amino acid sequences of human, monkey, pig, cow, mouse, and hamster nectin-1 revealed 90–98% identity in the ectodomain (Menotti *et al.,* 2000; Milne *et al.,* 2001; Shukla *et al.,* 2000). In addition, mouse and pig forms of nectin-1 have been shown to mediate the entry of HSV-1, HSV-2, PRV, and BHV-1 and to bind various forms of gD encoded by these viruses (Menotti *et al.,* 2000; Milne *et al.,* 2001; Shukla *et al.,* 2000). This ability of human and animal alphaherpesvirus gDs to recognize conserved features of the human and animal forms of nectin-1 explains in part their broad host ranges for entry and previous observations that expression of gD in certain cell types could interfere with entry of the homologous as well as heterologous alphaherpesviruses (Chase *et al.,* 1990, 1993; Geraghty *et al.,* 2000; Petrovskis *et al.,* 1988).

Nectin-1 is expressed in a variety of cell types, most notably in epithelial cells and neurons (Cocchi *et al.,*

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1998b; Geraghty *et al.,* 1998; Shukla *et al.,* 2000). Two isoforms, nectin-1α and nectin-1β, share identical ectodomains and herpesvirus entry activity but differ in their membrane spans and cytoplasmic domains due to differential splicing of the gene transcript (Cocchi *et al.,* 1998b; Geraghty *et al.,* 1998). Both nectin-1α and nectin-1β can engage in homotypic interactions through their ectodomains and nectin-1α localizes to regions of cadherin-based adherens junctions in epithelial cells through interactions of its cytoplasmic tail with PDZ domains in afadin, an actin-binding protein (Miyahara *et al.,* 2000; Tachibana *et al.,* 2000; Takahashi *et al.,* 1999).

The ectodomain of nectin-1 is composed of an N-terminal V-like domain and two C-like domains (Fig. 1). Several lines of evidence indicate that, for all the viruses mentioned above, the gD-binding region of nectin-1 is localized to the V-like domain. Soluble forms of HSV-1, HSV-2, and PRV gD bind efficiently to truncated or deleted forms of nectin-1 retaining only the V-like region and compete with each other for this binding (Cocchi *et al.,* 1998a; Connolly *et al.,* 2001; Krummenacher *et al.,* 1999). Monoclonal antibodies whose epitopes are located in the V domain can block the binding of HSV gD and infection (Cocchi *et al.,* 1998a; Krummenacher *et al.,* 2000). Also, nectin-1 deleted for the two C-like domains retains the ability to mediate HSV entry, albeit with reduced efficiency (Cocchi *et al.,* 1998a). Finally, expression of full-length gD encoded by HSV-1, HSV-2, PRV, or BHV-1 can interfere with entry of any of these viruses into cells expressing only nectin-1 as receptor, additional evidence that the different forms of gD compete for interaction with nectin-1 (Geraghty *et al.,* 2000).

To determine whether the two C-like domains of nectin-1 influenced gD binding and entry activity, genes encoding chimeric proteins were constructed. In one set of chimeras, portions of nectin-1 were replaced with homologous regions from nectin-2 (HveB, Prr2), a related protein with ability to mediate the entry of PRV, HSV-2, and Rid mutants of HSV-1, but not wild-type HSV-1 or BHV-1 (Lopez *et al.,* 2000; Warner *et al.,* 1998). Rid mutants of HSV-1 have amino acid substitutions at position 27 in gD (Dean *et al.,* 1994) that result in acquired ability to use nectin-2 for entry without effect on the use of nectin-1 for entry (Geraghty *et al.,* 1998; Montgomery *et al.,* 1996; Warner *et al.,* 1998). In the second set of chimeras, one or more domains of nectin-1 were fused to the two membrane-proximal Ig domains of CD4 (Wang and Springer, 1998), a protein with no herpesvirus entry activity and no detectable ability to bind to HSV gD (Whitbeck *et al.,* 1997). One of the chimeric molecules failed to bind to soluble forms of gD encoded by any of the alphaherpesviruses tested and also failed to mediate their entry. The other chimeric molecules bound to all the gDs but two of them failed to exhibit the predicted viral entry activities. The results indicate that binding of gD is not sufficient for entry activity, that there are structural requirements for entry independent of gD binding, and that these requirements are different for the various alphaherpesviruses tested.

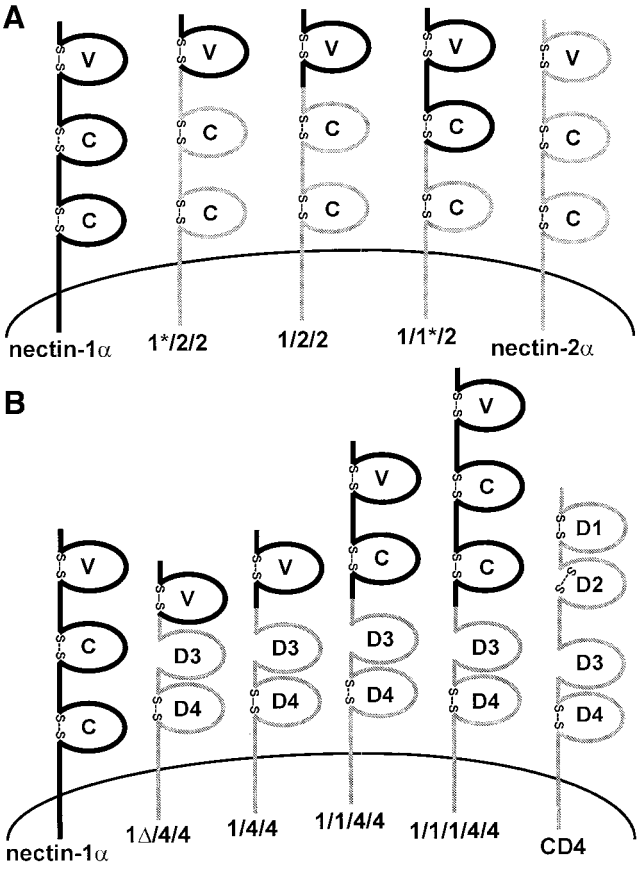

FIG. 1. Domain composition of nectin-1/nectin-2α chimeras and nectin-1/CD4 chimeras. (A) Nectin-1/nectin-2α chimeras in which regions of nectin-2α (thin gray lines) were replaced with the homologous regions from nectin-1 (thick black lines). The names given to the chimeras indicate whether the first, second, and third Ig-like domains match the sequence of nectin-1 or nectin-2. The name 1*/2/2 indicates that most of the nectin-2 V domain, up to the second conserved Cys, was replaced with the nectin-1 sequence, whereas, in 1/2/2, all of the nectin-2 V domain was replaced with the nectin-1 sequence. (B) Nectin-1/CD4 chimeras in which one (1/4/4), two (/1/1/4/4), or all three (1/1/1/4/4) of the Ig domains from nectin-1 were fused to the hinge region just upstream of the two membrane-proximal Ig domains of CD4. In chimera 1Δ/4/4 only part of the nectin-1 V domain, up to and including the second conserved Cys, was fused to CD4. Nectin-1 sequence is indicated by the thick black lines and CD4 sequence by the thin gray lines. The characteristic disulfide bonds predicted to be present in most Ig-like domains are indicated by "S-S." V, variable-like domain; C, constant-like domain; D, Ig-like domain. Drawing not to scale.

RESULTS

Construction of plasmids encoding nectin-1 chimeras

Figure 1A presents diagrams of the chimeric molecules formed by replacing domains of nectin-1α with homologous domains of the related nectin-2α. Although the nectin-1α open reading frame is larger than that for

nectin-2α (517 amino acids compared with 479 amino acids) and alignment reveals only about 35% identity in the ectodomains, the Ig domain organization is similar for the two proteins, so that chimeras could be constructed to retain the domain organization of both parental molecules. Chimera 1*/2/2 has most of the V-like domain of nectin-1 joined after the second conserved Cys to nectin-2α at the equivalent amino acid. Chimera 1/2/2 has the entire V-like domain of nectin-1 joined to the first C-like domain of nectin-2α midway between the second and third conserved Cys residues. Chimera 1/1*/2 has the entire V-like domain and most of the first C-like domain of nectin-1 joined after the fourth conserved Cys to nectin-2α at the equivalent amino acid. Figure 1B depicts the nectin-1 chimeras with CD4 in which one (1/4/4), two (1/1/4/4), or three (1/1/1/4/4) of the Ig domains of nectin-1 were fused to a hinge region in CD4 located N-terminal to the two membrane-proximal Ig domains. Chimera 1Δ/4/4 differs from chimera 1/4/4 in that a portion of the V-like domain of nectin-1 was deleted, namely, the amino acids following the second conserved Cys.

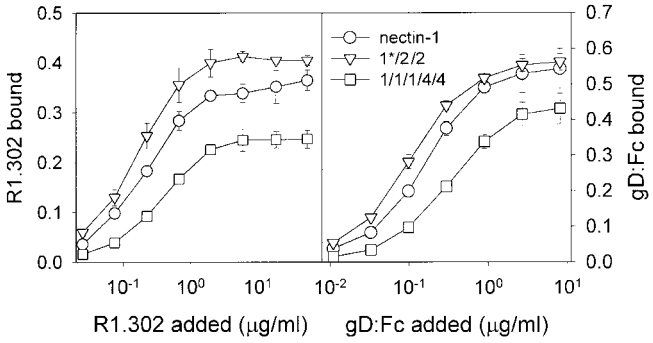

FIG. 2. Binding of an anti-nectin-1 mAb and wild-type HSV-1 gD:Fc to CHO cells expressing nectin-1 or two of the chimeric receptors. CHO cells were transfected with plasmids expressing the receptors indicated, replated in 96-well plates, and incubated in triplicate with serial dilutions of the mAb R1.302 or HSV-1 gD:Fc. The cells were then washed and fixed and incubated with biotinylated secondary antibodies and an avidin–HRP detection system. Similar results were obtained in two independent experiments done as indicated here and in numerous experiments done with single concentrations of mAb or gD:Fc known to give maximal binding. The symbols and error bars are the means and standard deviations, respectively, of triplicate determinations. The absence of error bars for mean values given in Figs. 2–7 is due to standard deviations too small to generate a visible error bar.

Cell surface expression and conformation of the nectin-1 chimeric receptors

To determine whether the chimeric proteins were expressed in cells and translocated to the cell surface with retention of gD-binding activity, Chinese hamster ovary (CHO) cells were transfected with plasmids expressing full-length nectin-1α or the various chimeric proteins and then tested by CELISA for the binding of monoclonal antibodies (mAbs) specific for nectin-1 and for the binding of soluble forms of gD.

One of the mAbs (R1.302) recognizes a conformational epitope within the V domain of nectin-1 (Cocchi *et al.,* 1998a; Krummenacher *et al.,* 2000). The others can bind to native or denatured nectin-1 and their epitopes have been mapped, by use of overlapping synthetic peptides, to sequences in the V domain from amino acids 70 through 114 (Krummenacher *et al.,* 2000). Three of the mAbs, R1.302, CK6, and CK8, can compete with HSV-1 gD for binding to nectin-1 (Cocchi *et al.,* 1998a; Krummenacher *et al.,* 2000). Soluble forms of HSV-1, HSV-1(Rid1), HSV-2, PRV, and BHV-1 gD were produced from plasmids in which sequences encoding the ectodomain of each viral protein were fused to sequences encoding the Fc region of rabbit immunoglobulin. For the CELISA, transfected cells were washed and incubated with the mAbs or gD:Fcs and then washed again and fixed prior to addition of secondary antibody and the detection system.

For all the chimeric receptors except one, as described more fully below, we found that the relative amounts of mAb R1.302 and gD:Fc bound to chimera in comparison with those bound to nectin-1α were similar, reflecting the relative amounts of properly folded V domain present at the cell surface. Figure 2 shows the concentration-dependent binding of R1.302 and HSV-1 gD:Fc to CHO cells transfected with plasmids expressing nectin-1α and two representative chimeric receptors. Reproducibly, we have found that both ligands bound as well or better to cells expressing chimera 1*/2/2 (and the other nectin-1/nectin-2 chimeras, as shown below) as to cells expressing nectin-1α, indicating that cell surface expression of these chimeras and certain critical aspects of their conformation in the V domain are probably nearly equivalent to that of nectin-1α. On the other hand, both ligands reproducibly bound somewhat less well to cells expressing chimera 1/1/1/4/4 and some of the other nectin-1/CD4 chimeras, indicating either somewhat reduced levels of expression or reduced affinity for the ligands. The results shown below for all the chimeric receptors depict the levels of various mAbs or gD:Fcs bound at saturating concentrations of ligand.

The results shown in Fig. 3 demonstrate that R1.302, CK6, and CK8 bound to cells expressing nectin-1α or the nectin-1/nectin-2α chimeras equally well, suggesting that none of these chimeras was impaired in expression, overall folding of the gD-binding region in the nectin-1 V domain, or translocation to the cell surface. The mAbs that bind to linear epitopes encompassing amino acids 70 to 114 (CK1, CK5, CK7, CK10, CK11), mostly in regions flanking the CK6 and CK8 epitopes, could bind to chimeras 1/2/2 and 1/1*/2 as well as or better than to nectin-1α but had reduced binding to chimera 1*/2/2, suggesting that this chimera did not have precisely that same conformation in the V domain as authentic nectin-1α.

Figure 4 presents similar results for the nectin-1/CD4

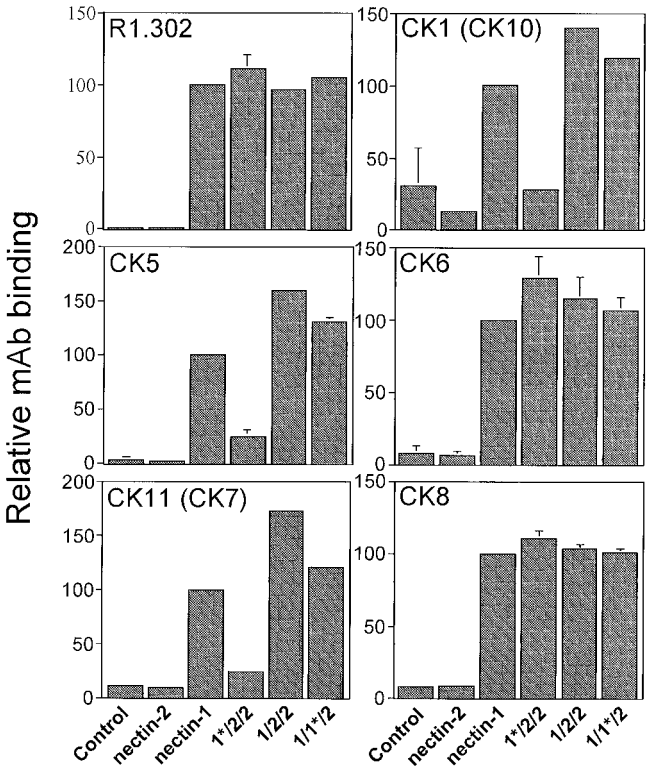

FIG. 3. Binding of anti-nectin-1 mAbs to CHO cells expressing nectin-1α or the nectin-1/nectin-2α chimeric receptors. CHO-K1 cells were transfected with plasmids expressing the molecules indicated or with control plasmid and then replated in 96-well plates. The cells were incubated in triplicate with the mAbs indicated, at 1:500 dilutions of ascites fluid. The cells were then washed and fixed and the bound mAbs detected as described in the text and the legend to Fig. 2. Within each experiment, the means of the triplicate determinations were expressed as a percentage of the mean obtained for the nectin-1α transfection (which ranged from 0.134 to 0.198 OD at 370 nm). The experiments were performed at least three times and the mean values plus standard deviations for the combined results are depicted. Each panel shows the results obtained with the mAb indicated. CK7 and CK10 (in parentheses) bind to epitopes that overlap those of CK11 and CK1, respectively (Krummenacher *et al.,* 2000), and gave results very similar to those shown for CK11 and CK1.

chimeric molecules. At least five of the mAbs that recognize linear epitopes (CK1, CK5, CK7, CK10, and CK11) bound almost as well to three of the chimeras (1Δ/4/4, 1/4/4, 1/1/1/4/4) as to nectin-1α, indicating that there is near-equivalent expression and translocation to the cell surface. Chimera 1/1/4/4 bound all of the antibodies at 50–75% of the amounts bound to nectin-1α, probably because this chimera exhibited somewhat reduced levels of expression or transport to the cell surface. The antibody that competes best with gD for binding to nectin-1 (R1.302) bound well to chimeras 1/4/4, 1/1/1/4/4, and probably also to 1/1/4/4 (assuming less cell surface expression) but failed to bind to chimera 1Δ/4/4, suggesting that the gD-binding region is not properly folded. CK6 and CK8, which also bind to a region of nectin-1 overlapping the gD-binding site, had reduced binding to all of the chimeras, particularly 1Δ/4/4 and 1/1/4/4/, but exhibited better binding to the former chimera than did R1.302. These results, taken together, suggest that chimeras 1/4/4 and 1/1/1/4/4 were expressed well, assumed a nearly normal conformation in the nectin-1 V domain, and were transported to the cell surface, that 1/1/4/4 was not so well expressed or transported, and that 1Δ/4/4 was probably improperly folded, particularly in the gD-binding region.

To test directly for gD-binding activity, CHO cells were transfected with plasmids expressing nectin-1α or each of the chimeric receptors and then incubated with gD:Fcs derived from HSV-1, HSV-1(Rid1), HSV-2, PRV, and BHV-1. After this incubation, the live cells were washed and fixed prior to addition of a secondary anti-rabbit antibody and a detection system for CELISA. The results shown in Fig. 5 demonstrate that all forms of gD:Fc bound as well or better to the nectin-1/nectin-2α chimeras as to nectin-1α (data not shown for HSV-2 gD:Fc). All forms of gD:Fc also bound to two of the nectin-1/CD4 chimeras, namely, 1/4/4 and 1/1/1/4/4, at about 70–95% of the levels bound to nectin-1α. The reduced binding of the gD:Fcs to chimera 1/1/4/4 is consistent with the reduced binding of

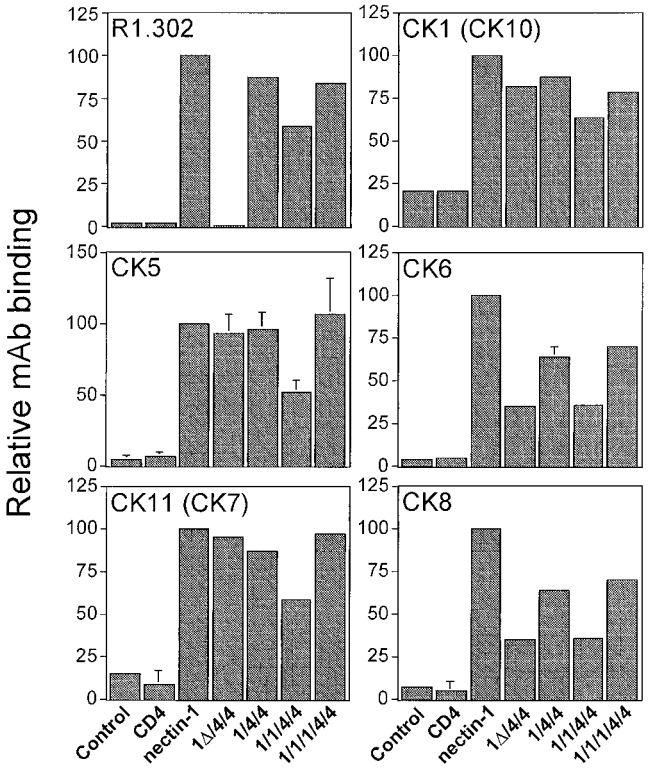

FIG. 4. Binding of anti-nectin-1 mAbs to CHO cells expressing nectin-1α or the nectin-1/CD4 chimeric receptors. CHO-K1 cells were transfected with plasmids expressing the molecules indicated or with control plasmid and then replated in 96-well plates. The cells were incubated in triplicate with the mAbs indicated. The mAb-binding assay was performed and the results are presented exactly as described in the legend to Fig. 3. The positive control values for binding of the mAbs to cells expressing nectin-1α ranged from 0.136 to 0.247 OD at 370 nm.

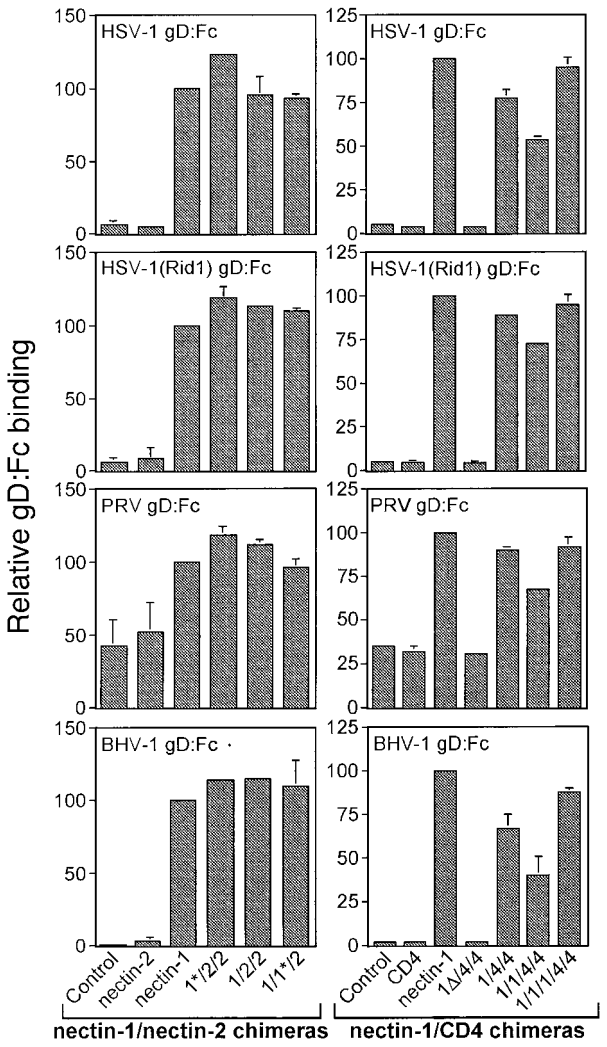

FIG. 5. Binding of various gD:Fcs to CHO cells expressing parental or chimeric receptors. CHO-K1 cells were transfected with plasmids expressing the molecules indicated or with control DNA and replated in 96-well plates. The next day the cells were incubated in triplicate with the various forms of alphaherpesvirus gD:Fc indicated and then washed and fixed and the bound gD:Fcs detected as indicated in the text and legend to Fig. 2. Within each experiment, the means of the triplicate determinations were expressed as a percentage of the mean obtained for the nectin-1α transfection (which ranged from 0.2 to 1.6 OD at 370 nm). The experiments were performed at least three times and the mean values plus standard deviations for the combined results are depicted. Results similar to those shown for HSV-1 gD:Fc were also obtained with HSV-2 gD:Fc (data not shown).

all the mAbs, probably resulting from reduced cell surface expression. None of the gD:Fcs was capable of binding to chimera 1Δ/4/4 at levels above background, consistent with the inability of R1.302 to bind. These findings, coupled with the fact that other mAbs bound well to chimera 1Δ/4/4, indicate that this protein is expressed on the cell surface in amounts comparable to the other chimeras but is misfolded in the V domain so that gD cannot bind. We recognize that the absence of significant binding of HSV-1(Rid1) and PRV gD:Fcs to parental nectin-2α is inconsistent with the ability of this receptor to mediate the entry of HSV-1(Rid1) and PRV. There are probably at least two explanations for this anomaly. The affinity of binding of appropriate forms of soluble gD to nectin-2α appears to be low (Connolly *et al.,* 2001; Lopez *et al.,* 2000). Also, although both nectin-1 and nectin-2 engage in homotypic interactions through their V domains (Miyahara *et al.,* 2000), preliminary evidence suggests that nectin-2α, but not nectin-1α, quantitatively localizes at regions of cell contact in transfected CHO cells (M. Yoon and P. G. Spear, unpublished studies). Soluble gD, but not virus, may fail to gain access to the V domain of nectin-2α under these conditions.

Entry activities of the nectin-1 chimeric receptors

Because CHO cells are normally resistant to entry of the alphaherpesviruses under study here, they could be used to assess the entry activities of the chimeric receptors as well as their expression and cell surface display of binding determinants. CHO cells were transfected with nectin-1α, nectin-2α, CD4, or each of the chimeric molecules and then tested for susceptibility of the cells to entry of wild-type HSV-1, HSV-1(Rid1), PRV, and BHV-1. The transfected cells were exposed to various concentrations of recombinant forms of these viruses, each expressing β-galactosidase from an inserted reporter gene. Enzyme activity was quantitated at 6 h after the addition of virus to measure the gene expression occurring immediately after viral entry.

Results obtained with the nectin-1/nectin-2α chimeras are shown in Fig. 6. As expected, nectin-1α mediated entry of all the viruses tested, whereas nectin-2α mediated entry of PRV and HSV-1(Rid1) only. Replacement of the entire V domain or more of nectin-2α with the homologous regions from nectin-1, in chimeras 1/2/2 and 1/1*/2, yielded receptors capable of mediating the entry of all four viruses with efficiencies as high as or higher than that observed for nectin-1α. Thus, the V domain of nectin-1 is all that is required for full entry activity for all four alphaherpesviruses, at least when this domain is linked to the two C domains of either nectin-1α or nectin-2α. A previous report documented the importance of the nectin-1 V domain for HSV-1 entry by showing that at least some entry activity was retained by a form of nectin-1β from which both C domains had been deleted (Cocchi *et al.,* 1998a). The third nectin-1/nectin-2α chimera, 1*/2/2, exhibited full entry activity for BHV-1 but was unexpectedly impaired in ability to mediate entry of HSV-1 and HSV-1(Rid1), particularly at low doses of virus, despite the fact that chimera 1*/2/2 bound all forms of gD:Fc very well (Fig. 5) and bound HSV-1 gD:Fc as well as did nectin-1α at all concentrations tested (Fig. 2). Thus, the amino acids C-terminal of the second con-

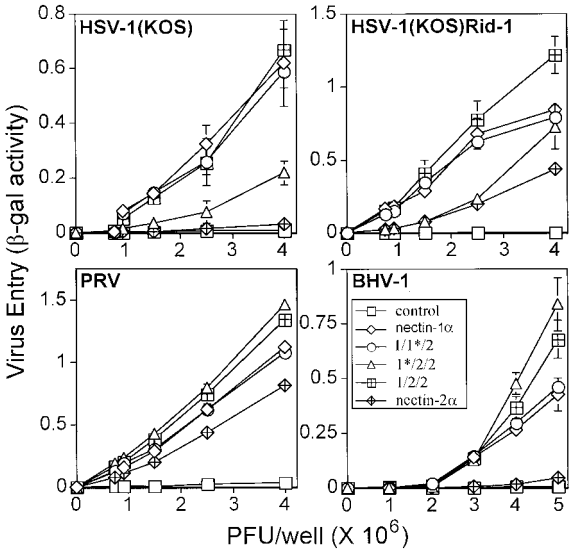

FIG. 6. Alphaherpesvirus entry activity of the nectin-1/nectin-2α chimeric receptors. CHO-K1 cells were transfected with plasmids expressing the proteins indicated or with a control plasmid and then replated in 96-well plates. The next day the cells were inoculated with HSV-1, HSV-1(Rid1), PRV, or BHV-1 recombinants expressing β-galactosidase. Six hours after inoculation, cells were lysed and β-galactosidase activity was determined as a measure of virus entry. The assays were performed in triplicate and repeated three times with similar results. The mean values plus standard deviations for a representative experiment are depicted.

served Cys in nectin-1α, in a region forming part of the putative G and G′ strands of the V-like Ig fold (Krummenacher *et al.,* 2000), appear to influence entry of HSV-1. It should be noted that all the parental and chimeric receptors mediated efficient entry of PRV, which provided assurance that critical features important to the activity of each parental molecule had not been destroyed by the gene fusions.

Results obtained with the nectin-1/CD4 chimeras are shown in Fig. 7. Chimera 1Δ/4/4 failed to mediate entry of any of the viruses tested, consistent with its inability to bind any of the gD:Fcs (Fig. 5) and with the body of evidence that gD binding is associated with alphaherpesvirus entry activity (reviewed by Spear *et al.,* 2000). The other chimeras, 1/4/4, 1/1/4/4, and 1/1/1/4/4, were almost as efficient as nectin-1α at mediating entry of PRV. Chimera 1/1/1/4/4, however, failed to mediate entry of any of the other viruses despite its ability to bind the gD:Fcs derived from these viruses. Chimeras 1/4/4 and 1/1/4/4 had equivalent entry activities for the HSV-1 strains but were somewhat less efficient than nectin-1α. Chimera 1/4/4 had partial activity for entry of BHV-1, while 1/1/4/4 was much less effective. Thus, PRV, HSV-1, and BHV-1 differ in ability to use these chimeras for entry despite the similarity in results obtained when the gD:Fcs derived from each virus were tested for binding to the chimeric receptors (Fig. 5).

DISCUSSION

An important conclusion of this study is that binding of alphaherpesvirus gD to a cell surface receptor does not necessarily result in viral entry. Two of the chimeric receptors, 1*/2/2 and 1/1/1/4/4, bound to all forms of alphaherpesvirus gD available for this study, apparently as well as did nectin-1α, but yet 1*/2/2 was impaired for the entry of HSV-1 and 1/1/1/4/4 failed to mediate entry of HSV-1 or BHV. Also, the ability of these chimeric receptors to mediate alphaherpesvirus entry was highly virus-specific. Chimera 1/1/1/4/4 was a fully functional entry receptor for PRV, as was 1*/2/2 for PRV, and BHV-1.

Comparisons of mAb binding to nectin-1α and chimeras 1*/2/2 and 1/2/2 revealed that 1*/2/2 is altered in conformation in the V domain even though it is apparently not altered in gD-binding activity (Figs. 2 and 5). Although the mAbs that can compete with gD for binding to the V domain of nectin-1 exhibited similar levels of binding to nectin-1 and both chimeras, other mAbs whose epitopes have been mapped to a region spanning amino acids 70–114 had reduced binding to chimera 1*/2/2. The differences in amino acid sequence between chimeras 1*/2/2 and 1/2/2 lie outside the epitopes for these mAbs, between amino acids 131 and 143, as shown in Fig. 8. This region encompasses the putative G and G′ β-strands of the V domain extending to the junction between the V and C domains. A site for the addition of an N-linked glycan is found in this region of 1/2/2 but is absent from 1*/2/2. Moreover, differences in the amino acid sequence shown in Fig. 8 are likely to affect interaction with other β-strands. These differences must influence the binding of some of the mAbs and the

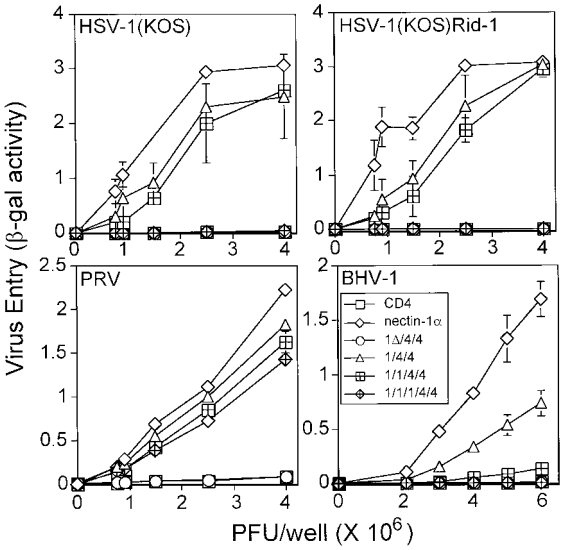

FIG. 7. Alphaherpesvirus entry activity of the nectin-1/CD4 chimeric receptors. CHO-K1 cells were transfected with plasmids expressing the proteins indicated or with a control plasmid and then replated in 96-well plates. The next day viral entry assays were performed and the results are presented as described in the legend to Fig. 6.

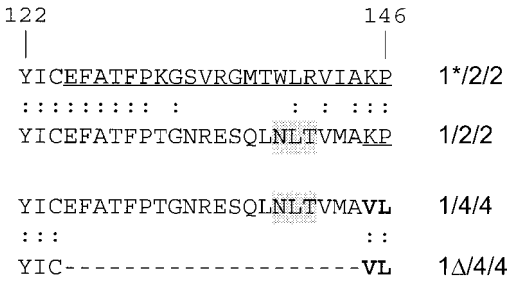

FIG. 8. Differences in amino acid sequence between the nectin-1/nectin-2α chimeras, 1*/2/2 and 1/2/2, and the nectin-1/CD4 chimeras, 1/4/4 and 1Δ/4/4. Numbering is from the initiator Met. Portions of each chimera derived from nectin-2α (top set) are underlined and portions derived from CD4 (bottom set) are in bold-faced type. Positions with identical amino acids are marked with double dots. The potential site for addition of an N-linked glycan in nectin-1 is shaded.

ability of 1*/2/2 to serve as an efficient entry receptor for HSV-1, but have little or no effect on binding of all the gD:Fcs tested, on binding of mAbs that compete with gD for binding, or on entry activity for PRV or BHV-1. Thus, the entry activity of a receptor depends not just on binding of gD but on structural features of the receptor in addition to the gD-binding domain.

Possibly, binding of gD induces conformational changes in both gD and receptor, postbinding events that depend in part upon the region altered in chimera 1*/2/2 and contribute to activation of the fusogenic activity of the viral glycoproteins required for entry. Alternatively, there may be multiple gD-binding sites on the nectin-1 V domain necessary to promote efficient viral entry. The CELISA assay may detect an initial or simple binding of the soluble gD:Fc to the V-like domain of nectin-1α and not distinguish between that initial interaction and a more complex interaction with gD that is required for efficient virus entry. Whatever the explanation, our results suggest that chimera 1/2/2 has all the features of nectin-1 that make it a functional receptor for the viruses studied here, whereas 1*/2/2 has the appropriate gD-binding domain for all these viruses and other features required for postbinding events leading to entry of PRV and BHV-1, but not HSV-1, or has features that impede the entry of HSV-1.

The inability of chimera 1Δ/4/4 to mediate entry of any of the alphaherpesviruses is explained readily by its failure to bind any of the alphaherpesvirus gDs. Deletion of the C-terminal portion of the V domain that extends from the second conserved Cys (Fig. 8) must have caused misfolding of the V domain so that gD binding was lost. Comparisons of mAb binding and gD:Fc binding to nectin-1α and chimera 1/1/1/4/4 suggest that the V domain of this chimera is properly folded and is functional for binding to all the alphaherpesvirus gD:Fcs tested here. Yet this molecule cannot serve as an entry receptor for HSV-1 or BHV-1 even though it is functional for PRV entry. Because nearly the entire ectodomain of nectin-1 is fused to CD4 in this chimera, it seems likely that conformation of the nectin-1 V domain is preserved. We cannot be certain how far this molecule extends from the plasma membrane but perhaps it extends too far to be an efficient entry receptor for some of the alphaherpesviruses. If distance of the nectin-1 gD-binding region from the plasma membrane is the explanation, why is PRV able to use chimera 1/1/1/4/4 for entry? There is as yet no answer to this question but it should be noted that significant amounts of PRV gD:Fc can bind to CHO cells even in the absence of transfected entry receptors (Fig. 5). Although CHO cells are resistant to PRV entry in the absence of a transfected entry receptor, it is possible that PRV entry can result from the synergistic effects of gD binding to a putative endogenous CHO receptor as well as to 1/1/1/4/4.

The results presented here confirm and extend previous reports that localized the binding sites for HSV-1, HSV-2, and PRV gDs to the V domain of nectin-1 (Cocchi *et al.,* 1998a; Connolly *et al.,* 2001; Krummenacher *et al.,* 2000, 1999). We show here that the binding site for BHV-1 gD is also localized to the V domain. The binding of HSV-1, HSV-2, PRV, and BHV-1 gD:Fcs to chimeric receptors depended only on the presence of the V domain from nectin-1. Moreover, each of the alphaherpesvirus gDs compete with each other for binding to nectin-1α (Connolly *et al.,* 2001; Geraghty *et al.,* 2000). Thus, the regions on nectin-1 to which the alphaherpesvirus gDs bind are overlapping but not necessarily identical.

The two isoforms of nectin-1 differ in their transmembrane domains and cytoplasmic tails but are identical in their Ig-like domains. Both are functional for alphaherpesvirus entry and most of the nectin-1/nectin-2 and nectin-1/CD4 chimeras described here are also functional for entry. Therefore, the structure of the transmembrane region and cytoplasmic tail is not important for alphaherpesvirus entry. In fact, deletion of the cytoplasmic tail from nectin-1α has no effect on entry activity (R. J. Geraghty and P.G. Spear, unpublished results). The features of nectin-1 that *are* important for alphaherpesvirus entry include the gD-binding region of the V domain and a region near the C terminus of the V domain in which certain amino acid substitutions do not affect gD binding but impair HSV-1 entry. It appears that molecules carrying the nectin-1 V domain work best as entry receptors when the V domain is fused to two Ig-like domains, as in nectin-1α itself or chimeras 1/2/2 or 1/1*/2 or less well in 1/4/4. Deletion of the two C domains of nectin-1 reduced the efficiency of entry (Cocchi *et al.,* 1998a), as did generation of chimeras with the nectin-1 V domain fused to 4 Ig-like domains. Because the amino acid sequence and alphaherpesvirus entry activity of nectin-1 are highly conserved among mammals, we predict that the structural requirements for alphaherpesvirus entry defined here for human nectin-1 will also be relevant to some of the animal forms of nectin-1.

MATERIALS AND METHODS

Cells and viruses

CHO-K1 cells were provided by J. Esko (University of California, San Diego) and were grown as previously described (Montgomery *et al.,* 1996). PEAK cells (Edge Biosystems), which are human embryonic kidney cells that express SV40 T antigen, were grown in DMEM supplemented with 10% fetal bovine serum and antibiotics. The β-galactosidase–reporter viruses have been described previously, HSV-1(KOS)tk12 and HSV-1(KOS)Rid1/tk12 (Warner *et al.,* 1998), PRV(Kaplan)gH$^-$ (Babic *et al.,* 1996) provided by T. Mettenleiter (Federal Research Centre for Virus Diseases of Animals, Insel Riems, Germany), and BHV-1(Cooper)v4a (Miller *et al.,* 1995) provided by L. Bello (University of Pennsylvania). The HSV strains were propagated and titered on Vero cells, PRV(Kaplan)gH$^-$ was propagated and titered on gH-expressing VeroSW78 cells, and BHV-1(Cooper)v4a was propagated and titered on MDBK cells.

Plasmids

All expression plasmids used the human cytomegalovirus (CMV) immediate-early promoter for efficient protein expression in eukaryotic cells. The nucleotide sequence was determined for relevant portions of all plasmids constructed using PCR, to guard against mutations arising during amplification. All PCR amplifications were performed using a step-down procedure (Geraghty *et al.,* 1998). The CD4 expression plasmid pBG53 was constructed by amplifying the CD4 open reading frame in pT4B (Maddon *et al.,* 1985) (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) with primers 5′CD4 (5′CGGGATCCATGAACCGGGGAGTCCCTTTTAGG) and 3′CD4 (5′CGTGGATCCGAAATGGGGCTACATGTCTTCTG), digesting the product with *Bam*HI, and ligating the fragment into the expression vector pMN104 (Montgomery *et al.,* 1996) digested with *Bam*HI. The templates for the PCR reactions described below were pBG38 (Geraghty *et al.,* 1998) to generate nectin-1α sequences, pMW20 (Warner *et al.,* 1998) to generate nectin-2α sequences, and pT4B to generate CD4 sequences.

Plasmids expressing receptor chimeras. The receptor chimera open reading frames were created by PCR. Briefly, for each chimera the nectin-1α sequences and the nectin-2α or CD4 sequences were amplified in separate reactions. One of the primers used in the nectin-1α amplifications contained nucleotides (18 to 20 bases) on its 5′ end that matched nectin-2α or CD4 sequences. Similarly, one of the primers used in the nectin-2α or CD4 amplifications had nucleotides on its 5′ end that matched the appropriate nectin-1α sequence. The respective amplification products were purified and combined in a final PCR reaction using the upstream flanking primer from the nectin-1α reaction and the downstream flanking primer from the nectin-2α or CD4 reaction. This product was then purified, digested with restriction endonucleases, and inserted into pcDNA3 under control of the CMV promoter. The plasmid names and amino acid composition for each chimera are indicated in Table 1.

TABLE 1

Plasmids Constructed to Express Chimeric Receptors

Plasmid	Chimera	Amino acid composition[a]
pBG48	1*/2/2	Nectin-1α 1–124 fused to nectin-2α 141–479
pBG71	1/2/2	Nectin-1α 1–144 fused to nectin-2α 161–479
pBG39	1/1*/2	Nectin-1α 1–226 fused to nectin-2α 239–479
pBG56	1Δ/4/4	Nectin-1α 1–124 fused to CD4 201–458
pBG59	1/4/4	Nectin-1α 1–144 fused to CD4 201–458
pBG69	1/1/4/4	Nectin-1α 1–252 fused to CD4 201–458
pBG63	1/1/1/4/4	Nectin-1α 1–337 fused to CD4 201–458

[a] Amino acid numbering starts with the initiator methionine residue. The GenBank Accession numbers are AF060231 for nectin-1α, AF058448 for nectin-2α, and M12807 for CD4.

Soluble gD-expressing plasmids. The plasmid expressing the HSV-1(KOS) gD:Fc fusion protein, pBG64, has been described. To create the HSV-1(KOS)Rid1 gD:Fc expression plasmid named pAF1, pCJ1 (Geraghty *et al.,* 2000) was digested with *Hin*dIII and *Ppu*MI and the fragment containing the portion of the gD gene encompassing the Rid1 mutation was ligated to the larger fragment obtained by digesting pBG64 with *Hin*dIII and *Ppu*MI. To create the PRV, BHV-1, and HSV-2 gD:Fc expression plasmids, each gD extracellular domain was amplified using PCR, and the PCR product digested with *Hin*dIII and *Eco*RI and ligated to the larger fragment obtained by digesting pBG64 with *Hin*dIII and *Eco*RI, such that the HSV-1(KOS) gD ectodomain was replaced by the appropriate gD ectodomain from another virus, fused in frame to the rabbit IgG Fc region. The primers used to create the PRV gD:Fc expression plasmid, pBG73, were PgDFc1 (5′ATGAATTCGCGGCTCCGCGGGCTGCGGC) and PRVHIII (5′AGCTAAGCTTATGCTGCTCGCAGCGCTATT) and the template was the PRV gD expression plasmid, pCMVgD (Gerdts *et al.,* 1997). The primers used to create the BHV-1 gD:Fc expression plasmid pBG74 were BgDFc1 (5′ATGAATTCGCACGGCGTCGGGGGCCGCG) and BHVHIII (5′TAGGGAGACCCAAGCTTATGCAAGGGCCGACA) and the template was the BHV-1 gD expression plasmid, pBG58 (Geraghty *et al.,* 2000). The primers used to create the HSV-2 gD:Fc-expressing plasmid, pBG75, were HindIIIbest (5′CAAATGGGCGGTAGGCGTGTACGGTGGGAG) and 2gDRI (5′ATGAATTCGGATGATCAGGCCCGGGTTGC) and the template was pWM46. The plasmid pWM46 was created by digesting the HSV-2(333) gD-containing plasmid pWW65 (Muggeridge *et al.,* 1990) with *Hin*dIII and inserting the fragment containing the full-length HSV-2 gD into pcDNA3 digested with *Hin*dIII.

Soluble gD:Fc fusion proteins

To produce the gD:Fc proteins, PEAK cells were transfected with one of the gD:Fc-expressing plasmids by the calcium phosphate method (according to the Edge Biosystems protocol). The cells were incubated in DMEM containing 5% low-Ig calf serum, and 48 h later the culture supernatant was collected. The culture supernatants were clarified by low-speed centrifugation prior to use and the concentration of gD:Fc protein was determined by ELISA with reagents that detected the rabbit Fc region. A full description of the properties of these gD:Fc proteins will be published elsewhere (A. Fridberg, R. J. Geraghty, B. Lum, and P. G. Spear, manuscript in preparation).

Assays for binding of mAbs and gD:Fcs to cells (CELISA)

CHO-K1 cells were transfected with plasmids expressing nectin-1α, nectin-2α, CD4, or chimeric receptors or with control plasmid without insert (pcDNA3), using LipofectAMINE (Gibco-BRL) as recommended by the manufacturer. Each well of a 6-well plate received 5 μl of LipofectAMINE and 1.5 μg of plasmid DNA. After 24 h, the transfected cells were replated into 96-well dishes (approximately 4×10^4 cells per well). The next day, the cells were incubated with anti-nectin-1 mAbs in 50 μl of phosphate-buffered saline containing 3% bovine serum albumen (PBS/3% BSA) or with culture supernatants containing one of the gD:Fc molecules described above and diluted with PBS/3% BSA. After 30 min at room temperature, the cells were washed five times with PBS and fixed with 100 μl of 2% formaldehyde–0.2% glutaraldehyde for 10 min at room temperature. The cells were washed three times with PBS/3% BSA and incubated with biotinylated secondary antibodies against rabbit IgG (Sigma) for the gD:Fc molecules or mouse IgG (Sigma) for the mAbs at a 1:500 dilution in 100 μl PBS/3% BSA for 30 min at room temperature. Following the secondary incubation, the cells were washed five times with PBS and incubated with AMDEX streptavidin-conjugated horseradish peroxidase (Amersham) at 1:20,000 dilution in 100 μl of PBS/3% BSA/0.1% Tween 20 for 30 min at room temperature. Following tertiary incubation, the cells were washed five times in PBS/0.1% Tween 20 and incubated with 3,3′,5,5′-tetramethylbenzidine in phosphate-citrate buffer according to the manufacturer's instructions (Sigma). At various times after the addition of substrate, the plates were read at 370 nm in a Spectra Max 250 ELISA reader. The mAbs used included R1.302 (Cocchi *et al.,* 1998a) and CK1, CK5, CK6, CK7, CK8, CK10, and CK11 (Krummenacher *et al.,* 2000). Each binds to an epitope in the V domain of nectin-1.

Assay for viral entry

CHO-K1 cells were transfected as described above and replated in 96-well plates. The next day the cells were exposed to various dilutions of the viruses described above, in PBS containing 0.1% glucose and 1% calf serum. After 6 h, the cells were lysed and β-galactosidase activity was determined, as previously described (Montgomery *et al.,* 1996), as a measure of viral entry.

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